

**Remarks:**

The Office Action dated April 2, 2004 stated that a copy of the Hochuli *et al.* reference was not of record in the parent application. Applicants' records indicate that this reference was submitted with the original IDS in the parent file, but that the Office's records did not indicate as such. Applicants re-submitted the reference in a communication by facsimile on November 3, 2000 in the parent application, and Applicants' representative received a Form 892 from the Office by facsimile indicating that the reference was cited in the parent application, but that it might not be cited on the front page of the issued patent. Copies of those communications are re-presented herewith. In any case, Applicants' re-submit the Hoculi *et al.* reference again with this response.

Reconsideration of the application in view of the above amendments and following remarks is requested. Claims 11, 12, 18-23, 36, 38, 40 and 41 are now pending in the application. Claims 11, 12, 18, 20, 36, 38, 40, and 41 have been amended. Claims 1-10, 13-17, 24-35, 37, and 39 have been canceled.

Amendments to claims 11, 12, 18, 20, 36, and 38 require that the polypeptide molecule binds an integrin. Support for this claim can be found in the specification, for example, at page 54, lines 3-15, and the disclosure from page 63, line 7 to page 65, line 5. Amendments to claim 41 can be found on page 16, lines 27 to 29, and on page, 63 lines 31 to 34 of the instant specification.

The amendments to claim 18 obviate the Office's objection to claim 19 as being dependent from a rejected claim.

On the basis of the above amendments and remarks, Applicants believe that each rejection has been addressed and overcome. Reconsideration of the application and its allowance are requested. If for any reason the Examiner feels that a telephone conference would expedite prosecution of the application, the Examiner is invited to telephone the undersigned at (206) 442-6752.

Respectfully Submitted,



Robyn Adams  
Registration No. 44,495

Enclosures:

Amendment Fee Transmittal (in duplicate)

Application Serial No.:09/809,617  
Amendment dated: October 1, 2004  
Response to Office Action dated: April 2, 2004

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Petition for Extension of Time (in duplicate)  
Hochuli *et al.* reference  
Copies of communication in parent application dated November 3, 2000 and  
March 8, 2001  
Postcard



## FACSIMILE TRANSMITTAL SHEET

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ZymoGenetics, Inc.  
1201 Eastlake Avenue East  
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PLEASE DELIVER TRANSMISSION TO:

THIS TRANSMISSION FROM:

Name: Examiner Dr. Kathleen Kerr  
U.S. Patent and Trademark Office  
Office: Group Art Unit 1652

Name: Robyn Adams

Date: November 3, 2000

Fax #: (703) 308-0294

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**IF THERE ARE ANY PROBLEMS, PLEASE CALL (206) 442-6752**

Applicants : Paul O. Sheppard  
Serial No. : 09/351,414  
Filed : July 9, 1999  
For : DISINTEGRIN HOMOLOS

Examiner : Kerr, K.  
Art Unit : 1652  
Docket No.: : 98-29  
Date : November 3, 2000

Dear Examiner Kerr:

As per our phone conversation today attached is a copy of the Hochuli reference that was inadvertently left out of the amendment filed on November 3, 2000. Please call me if you have any questions.

Respectfully submitted,

Robyn Adams  
Registration No. P 44,495

# GENETIC APPROACH TO FACILITATE PURIFICATION OF RECOMBINANT PROTEINS WITH A NOVEL METAL CHELATE ADSORBENT

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We describe a general purification method for recombinant proteins based upon the selective interaction between a poly-histidine peptide, which is fused to the protein of interest, and a novel metal chelate adsorbent. The principle of the technique is illustrated with mouse dihydrofolate reductase. DNA elements coding for adjacent histidines were fused to the mouse dihydrofolate reductase gene. Subsequent expression in *E. coli* resulted in the production of hybrid proteins that could be purified by immobilized metal ion affinity chromatography, followed by removal of the histidine affinity peptide with carboxypeptidase A.

In recent years several genetic approaches to facilitate purification of heterologously-produced proteins have been described. Hybrid proteins were prepared by fusing the coding sequence of a protein of interest with the coding sequence for staphylococcal protein A, together with the sequence of a specific cleavage site<sup>1</sup>. Such fusion proteins could efficiently be purified by taking advantage of the specific binding of the protein A affinity tail to immobilized IgG. After purification of the fusion protein, the affinity tail was split off at the designed cleavage site.

Other gene fusion systems, based on fusion to  $\beta$ -galactosidase<sup>2</sup> and polyarginine<sup>3</sup> have been reported. The  $\beta$ -galactosidase hybrid proteins have been purified on a  $\beta$ -galactosidase-specific affinity resin and the polyarginine hybrid proteins on a cation exchange resin. Recently, we described a novel metal chelate adsorbent for immobilized metal ion affinity chromatography. This affinity resin, when charged with nickel ions ( $\text{Ni}^{2+}$ -NTA adsorbent), has a remarkable selectivity for proteins and peptides containing neighbouring histidine residues<sup>4</sup>.

In this paper we describe the production and subsequent purification by affinity chromatography on the  $\text{Ni}^{2+}$ -NTA adsorbent of hybrid proteins comprising the protein of interest and histidine affinity-peptides. The fusion concept is exemplified by mouse dihydrofolate reductase (DHFR). We prepared DNA elements that upon integration into expression vectors directed the

synthesis of DHFR derivatives comprising two to six histidine residues, either at the amino terminus or at the carboxy terminus. To investigate the potential of poly-histidine fusions for protein purification, their chromatographic behaviour was studied under a variety of conditions. In addition, the removal of the histidine affinity-peptide after purification of the fusion protein with carboxypeptidase A (CPA) is demonstrated.

## RESULTS

Cloning and expression of His/DHFR fusion proteins. Plasmid pDS78/RBSII (Fig. 1) was used for the construction of plasmids directing the expression of DHFR derivatives with adjacent histidine residues. This plasmid belongs to the pDS-family of plasmids<sup>5</sup> and contains the following elements: a. the regulatable promoter/operator element N250PSN250P29 (Bujard, H., Lanzer, M. unpublished), which is repressed in the presence of the *E. coli* lac repressor but can be induced by addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG); b. the synthetic ribosomal binding site RBSII; c. the coding region for mouse dihydrofolate reductase from cell line AT-3000<sup>6,7</sup> that had been altered by introduction of a BglII restriction site (due to this modification, plasmid pDS78/RBSII encodes a DHFR derivative with the genuine carboxy terminal amino acid Asp replaced by amino acids Gly-Ser-Arg-Ser-Val-Asn-Leu-Val); d. the transcriptional terminator  $\omega$  of phage lambda<sup>8</sup>; e. the promoter-free gene for chloramphenicol acetyltransferase with its genuine translational signals<sup>9</sup>; f. the transcriptional terminator T1 of the *E. coli* *trnB* operon<sup>10</sup> and g. the replication region and the gene for  $\beta$ -lactamase present in plasmid pBR322<sup>11</sup>. Due to the high efficiency of the expression signals the different vectors depicted in Fig. 1 can only be stably maintained when the plasmid pDM1.1, which expresses elevated levels of lac repressor, is present in the cells<sup>12</sup>.

For construction of plasmids p(His)<sub>n</sub>-DHFR (n = 2,3,4,5,6) adaptor fragments, encoding two to six adjacent histidines, were inserted into the BamHI site of plasmid pDS78/RBSII (Fig. 1). Plasmids pDHFR-(His)<sub>n</sub> (n = 2,3,4,5,6) were constructed by replacing the BglII/HindIII fragment of pDS78/RBSII by adaptor fragments encoding two to six histidines (Fig. 1).

*E. coli* cells containing plasmid pDM1.1 were transformed with the different plasmids represented in Figure 1 and subsequently grown in medium containing ampicillin (Ap) and kanamycin (Km) to select for the presence of the expression-plasmid (Ap) and plasmid pDM1.1 (Km). After induction with IPTG the cellular proteins of the

different cultures were analyzed by SDS-Page. As exemplified in Figures 2 and 4, the plasmid constructs directed the synthesis of the expected His/DHFR fusion proteins.

**Purification of the fusion proteins.** (a) *Chromatography in 0.05M phosphate buffer.* DHFR with two histidine residues at the carboxy terminus (DHFR-(His)<sub>2</sub>) produced with cultures of *E. coli* was extracted by sonication in 0.05M phosphate buffer pH 8 and purified by chromatography on the Ni<sup>2+</sup>-NTA column (Table 1). SDS-page and enzyme activity analysis demonstrate that DHFR-(His)<sub>2</sub> was retained at pH 8 on the adsorbent and the contaminating *E. coli* proteins were washed out. The fusion protein was eluted with a linear pH gradient from 8 to 5 (Fig. 2), yielding a product with a purity of at least 90% and a specific activity of 9 units/mg. When DHFR containing six histidine residues at the amino terminus ((His)<sub>6</sub>-DHFR) was extracted and purified in the phosphate buffer system, the fusion protein was retained on the affinity column but only about 10% was eluted. After addition of 6M guanidine hydrochloride (GuHCl) to the elution buffers, the protein was recovered completely (data not shown).

(b) *Cleavage of the -(His)<sub>2</sub> affinity-peptide with carboxypeptidase A (CPA).* CPA releases most rapidly amino acids with an aromatic or large aliphatic side chain from the carboxy-termini of polypeptide chains. When purified DHFR-(His)<sub>2</sub> was treated with CPA, two equivalents of His and one equivalent of Ser were released (Fig. 3). This result is in good agreement with the expected release of amino acids according to the carboxyterminal amino acid sequence

-----Lys-Gly-Ser-Arg-Ser-His-His

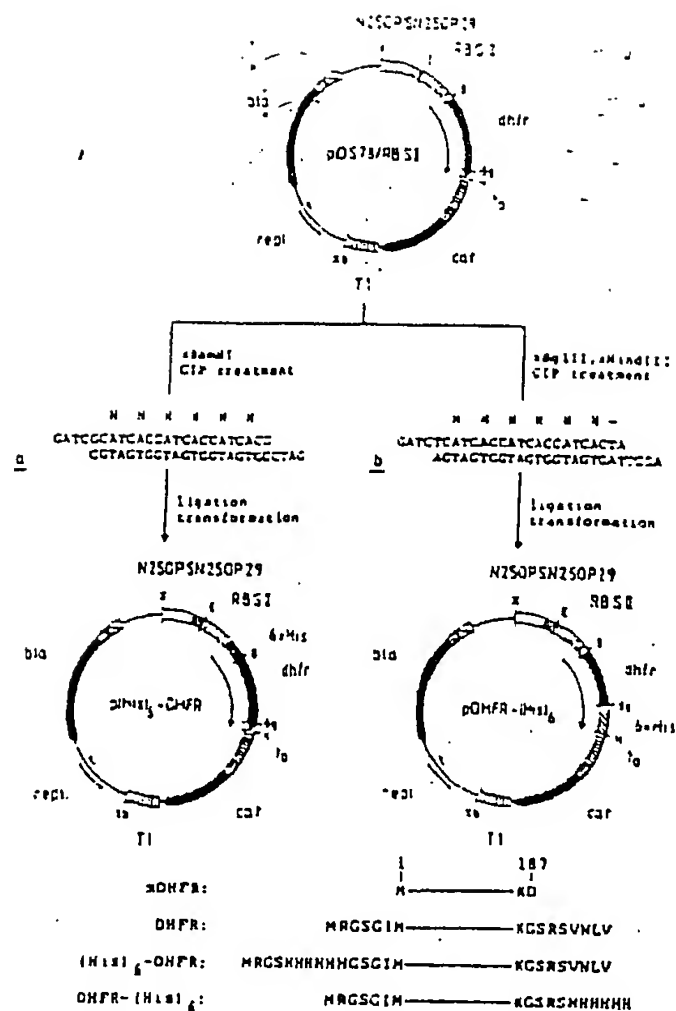
of the fusion protein, since Arg is expected to be digested very slowly with CPA. After the enzymatic cleavage, the reaction mixture was pumped without further treatment of the NTA column used for the purification of DHFR-(His)<sub>2</sub>. The protein was not retained on the affinity adsorbent, indicating that the -(His)<sub>2</sub> affinity-peptide was removed. The protein in the column flow-through was pooled and identified as DHFR by SDS-Page analysis (data not shown). The specific activity of the pooled DHFR was 9.8 U/mg (Table 1).

(c) *Chromatography in 6M guanidine hydrochloride.* DHFR with six histidine residues at the amino terminus ((His)<sub>6</sub>-DHFR) was extracted with 6M guanidine hydrochloride (GuHCl) from the *E. coli* cell paste and purified by column chromatography on the Ni<sup>2+</sup>-NTA adsorbent (Fig. 4). The crude extract was loaded without further purification onto the metal chelate adsorbent. The column was developed with a pH-step gradient. All buffer solutions contained 6M GuHCl throughout the chromatography. Collected fractions were dialyzed against water, lyophilized and analyzed. SDS-Page analysis of the crude extract and the purified protein shows that the fusion protein was retained on the column at pH 8 and, after washing out the contaminants, was eluted with a purity of at least 90%. Amino acid sequence determination (Edman-degradation) confirmed the sequence

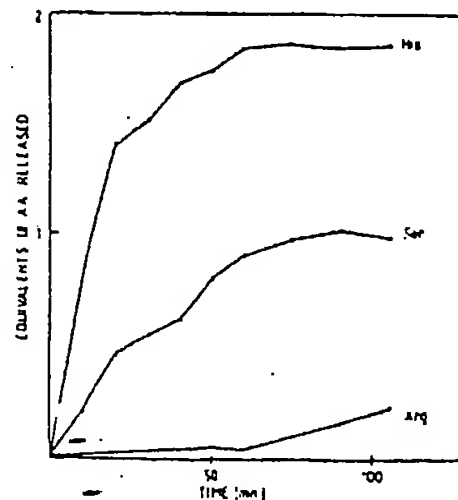
Met-Arg-Gly-Ser-His-His-His-His-His-Gly-Ser-Gly-Ile-Met---

of the purified fusion protein. Starting with 2g of biomass, 25 mg (His)<sub>6</sub>-DHFR with a specific activity of 2.3 units/mg were obtained. DHFR-(His)<sub>2</sub> extracted with 6M GuHCl was not retained on the Ni<sup>2+</sup>-NTA column equilibrated with extraction buffer. Most of the fusion protein was found in the column flow-through (data not shown).

In Table 2, the chromatographic behaviour of the two described and eight additional poly-histidine fusions, examined under the same conditions, are summarized.



**FIGURE 1** Plasmids used for the expression of His/DHFR fusion proteins. The upper part of the figure shows plasmid pDS78/RBSII and the construction of plasmids p(His)<sub>6</sub>-DHFR and pDHFR-(His)<sub>6</sub>. All plasmids contain the regulatable promoter/operator element N250PSN250P29 (□□) and the synthetic ribosomal binding site RBSII (□). Insertion of adaptor (a) into the BamHI site results in plasmid p(His)<sub>6</sub>-DHFR. Replacement of BglIII/HindIII fragment by adaptor (b) results in plasmid pDHFR-(His)<sub>6</sub>. The genes for β-lactamase, chloramphenicol acetyltransferase and mouse dihydrofolate reductase ( ) are denoted bla, cat and DHFR, respectively. Calf intestinal alkaline phosphatase is abbreviated with CIP. Transcriptional terminators ( ) of phage lambda and T1 of *rnaB* operon of *E. coli* are indicated by T<sub>0</sub> and T<sub>1</sub>. B, Bg, E, H, X and Xb denote cleavage sites for restriction enzymes BamHI, BglIII, EcoRI, HindIII, XhoI and XbaI. For the different plasmids, the coding region under control of N250PSN250P29 and RBSII is indicated by an arrow. The amino acid sequences of DHFR (encoded by plasmid pDS78/RBSII), (His)<sub>6</sub>-DHFR (plasmid p(His)<sub>6</sub>-DHFR) and DHFR-(His)<sub>2</sub> (plasmid pDHFR-(His)<sub>2</sub>) are displayed together with the amino acid sequence of mouse dihydrofolate reductase<sup>7</sup> (mDHFR) in the lower part of the figure.



**FIGURE 3** Digestion of DHFR-(His)<sub>2</sub> with CPA. The amount of released amino acids is plotted against time.

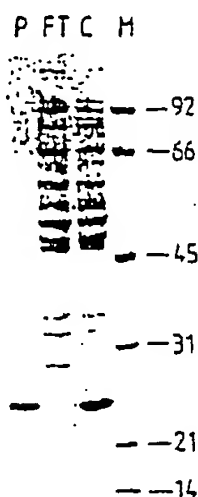
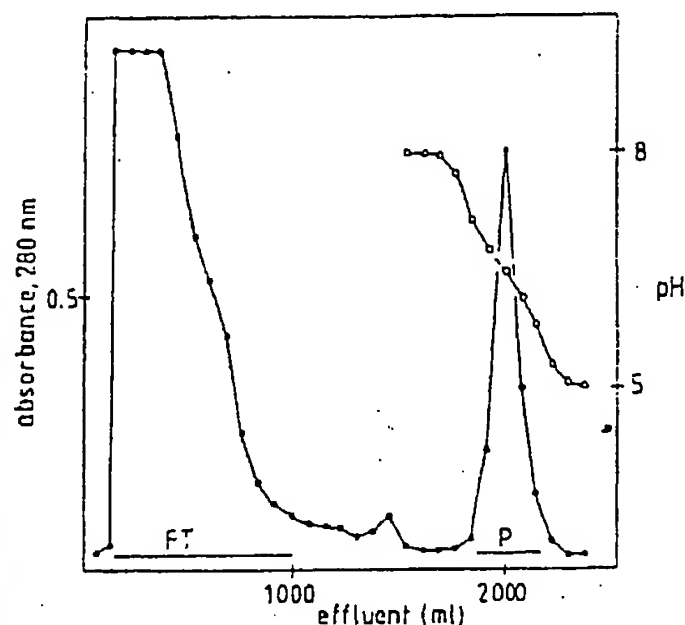
These results show that the affinity and elution behavior of the poly-histidine fusions are strongly dependent on the solvent system used throughout the chromatography. For the purification in 0.05M phosphate buffer the di-histidine affinity peptide at the carboxy terminus was most efficient, whereas for the recovery in 6M GuHCl the hexa-histidine affinity peptide gave the best result.

## DISCUSSION

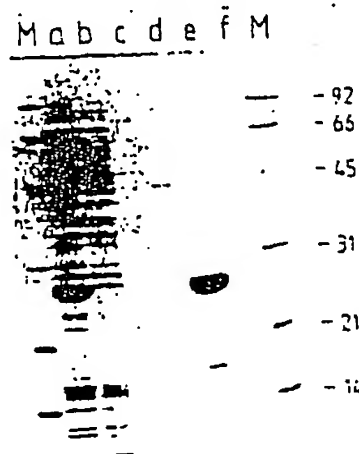
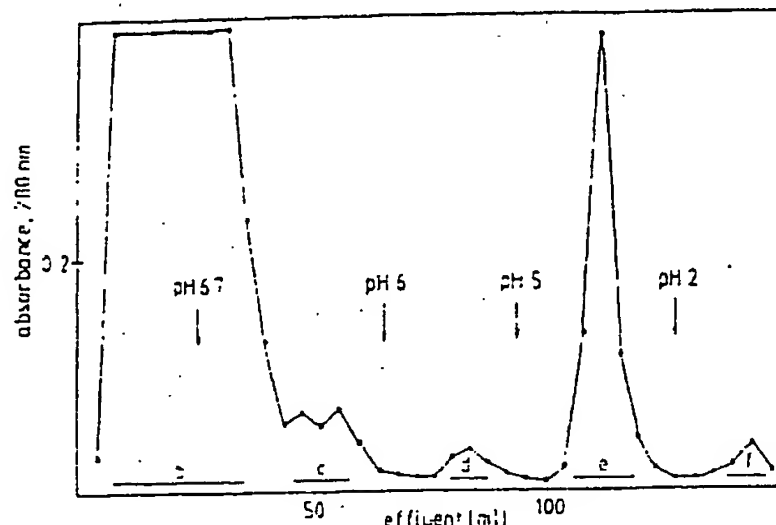
We have shown that fusion proteins comprising a protein of interest and a poly-histidine peptide can be purified very efficiently on a novel metal chelate adsorbent when charged with nickel ions ( $\text{Ni}^{2+}$ -NTA adsorbent). The efficiency of the poly-histidine peptide, as

exemplified with DHFR, is dependent on the solvent system used throughout the chromatography. DHFR-(His)<sub>2</sub> was recovered with a purity of >90% and 88% yield in 0.05M phosphate buffer, but the same protein did not bind to the  $\text{Ni}^{2+}$ -NTA column in the presence of 6M GuHCl. On the other hand (His)<sub>6</sub>-DHFR was purified very efficiently (purity >90%, yield: 90%) in 6M GuHCl, whereas this fusion protein bound too strongly in 0.05M phosphate buffer and could not be eluted completely. These results suggest that the di-histidine at the carboxy terminus for physiological buffers and the hexa-histidine—either at the carboxy or the amino terminus—for highly dissociating solutions are the affinity-peptides of choice.

Beside DHFR fusion proteins several surface antigens



**FIGURE 2** Chromatography of DHFR-(His)<sub>2</sub>. The extraction of (DHFR-His)<sub>2</sub> from *E. coli* is described in Table 1. 189 ml crude extract were loaded at a flow rate of 180 ml/h onto a  $\text{Ni}^{2+}$ -NTA column (5 × 16 cm), equilibrated with extraction buffer (0.05 M sodiumphosphate; pH 8; 0.1 M KCl; 0.1% Tween-20, 10  $\mu$ M PMSF). The column was washed with 1500 ml extraction buffer and then DHFR-(His)<sub>2</sub> was eluted with a 1000 ml pH gradient from 8 to 5 (phosphate buffer system). The column flow-through and eluted product were analyzed by SDS-Page. There is evidence that the contaminating protein in lane P is a fragment of DHFR-(His)<sub>2</sub>. (M) Standard molecular weight markers (C) *E. coli* extract containing DHFR-(His)<sub>2</sub> (FT) Column flow-through (P) Purified DHFR-(His)<sub>2</sub>.



**FIGURE 4** Chromatography of (His)<sub>6</sub>-DHFR. Two g *E. coli* cells were extracted with 10 ml 6M GuHCl, pH 8, for 1 h at 22°C. After centrifugation, the supernatant was pumped at a flow-rate of 30 ml/h onto a  $\text{Ni}^{2+}$ -NTA column (1.6 × 7.2 cm) equilibrated with extraction buffer. The column was developed with a pH-step gradient. The buffers contained 6M GuHCl and 0.1M sodiumphosphate. Collected fractions were dialyzed against water, lyophilized and analyzed by SDS-Page. The column was regenerated with 0.2M acetic acid (fraction f). The main contamination in the purified product (lane c) probably is a fragment of (His)<sub>6</sub>-DHFR. This protein is not present in the crude extract prepared with the *E. coli* M15 host. (M) standard molecular weight markers (a) *E. coli* extract containing (His)<sub>6</sub>-DHFR (b) column flow-through (c) fraction eluted at pH 6 (d) fraction eluted at pH 5.7 (e) purified (His)<sub>6</sub>-DHFR eluted at pH 5 (f) fraction eluted with 0.2M acetic acid.



of *Plasmodium falciparum* have successfully been purified using this principle<sup>1</sup>. Orienting experiments have shown that the purification protocol is also compatible with reducing agents as mercaptoethanol or dithiothreitol present in the extraction buffer. However as thiol groups can also form metal complexes, the affinity of the poly-histidine peptides for the Ni<sup>2+</sup>-NTA adsorbent is somewhat reduced in the presence of these reducing agents.

Recombinant proteins expressed in *E. coli* often are formed in an insoluble form. These refractor bodies can only be dissolved with detergents or highly concentrated dissociating agents as guanidine or urea. But these solutions are detrimental to most of the conventional chromatographic methods. The method of choice may be chromatography in 6M GuHCl on the Ni<sup>2+</sup>-NTA column in combination with the hexa-histidine fusion.

To split off the affinity peptide from the protein of interest, a specific chemical or enzymatic cleavage site may be introduced at the junction<sup>1</sup>. However, this method has limitations. If the desired protein already comprises the recognized amino acid or peptide sequence, the product will be degraded. In the described example with DHFR-(His)<sub>2</sub> we exploited the presence of an Arg at the penultimate position to the carboxy terminus. Since Arg is not digested by carboxypeptidase A, the histidine affinity-peptide as well as the carboxy terminal Ser were removed. From the above discussion it is evident that the method of cleavage must be determined on a case-by-case basis, after analysis of the amino acid sequence of the protein of interest. This consideration not withstanding, we believe the method described here will be applicable to the large-scale purification of a variety of heterologously-produced proteins.

#### EXPERIMENTAL PROTOCOL

**Cloning and expression of His/DHFR fusion proteins.** The oligonucleotides forming adapters a and b (Fig. 1) were synthesized chemically on controlled pore glass as solid support according to Bannwarth and Jaiza<sup>14</sup>, before they were phosphorylated in excess ATP using T4 poly-nucleotide kinase (Gibco-BRL). These adapters were cloned into the BamHI site or BglII/HindIII sites of plasmid pDS78/RBSII as outlined in Figure 1. The different plasmid constructs were transformed in *E. coli* M13 cells containing plasmid pDM1,1. The *E. coli* transformants were grown at 37°C in LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. At OD 600 nm ~0.7 isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2mM and the cells incubated for an additional 5 h., before harvesting by centrifugation.

**Purification of His/DHFR fusion proteins.** The biomass was recovered from the fermentation broth by centrifugation (4,000 × g, 10 min., 4°C) and the supernatant discarded. Generally 2-3 g of biomass were recovered from 1 litre of fermentation broth. Desintegration of the cells was performed either with 6M guanidine-hydrochloride in 0.1M sodium phosphate, pH 8 (10 g cells in 50 ml extraction buffer) for 1 h at 22°C or by sonification in 0.05M sodium phosphate buffer, pH 8, containing 0.1M potassium chloride, 0.1% Tween-20, 10 µM PMSF (10 g cells in 60 ml extraction buffer) with a 0.5 inch probe (Sonicator W-375 from Ultrasonic Inc. New York) for 15 min. at 0°C. After centrifugation (10,000 × g, 30 min., 4°C), the supernatant was directly pumped on a nickel NTA-chelate column equilibrated with extraction buffer. The metal chelating adsorbent charged with nickel ions (Ni<sup>2+</sup>-NTA adsorbent) was prepared as previously described<sup>1</sup>. For the chromatography in 6M guanidine the buffer system used was 0.1M sodium phosphate containing 6M guanidine-hydrochloride. The column was developed with a pH-step gradient (pH 8: load of the crude extract onto the column, pH 6.7 and 6: wash steps, pH 5: elution of the product). Collected fractions were dialyzed against water, lyophilized and analyzed by SDS-Page. The fusion protein extracted by sonification was chromatographed in phosphate buffer. After the loading of the crude extract the column was first washed with extraction buffer and then the product was eluted with a linear pH gradient from 8 (extraction buffer) to 5 (0.05M sodium phosphate containing 0.1M potassium chloride and 0.1% Tween-20).

Digestion of DHFR-(His)<sub>2</sub> with CPA. 60 mg of purified DHFR-(His)<sub>2</sub> were dialyzed against 0.05 M Tris-HCl buffer pH 8, and the protein incubated at room temperature with 6 mg of CPA from bovine pancreas (Serva, Feinbiochemica, Heidelberg). At the times indicated in Figure 3, 100 µl reaction mixture were sampled; the protein precipitated with trichloroacetic acid (25 µl) and the supernatant analyzed for amino acid content. After 105 min. the reaction mixture was pumped onto the Ni<sup>2+</sup>-NTA column equilibrated with 0.05M Tris/HCl buffer, pH 8. The flow through was pooled and assayed for DHFR activity (Table 1).

**Purification of mDHFR.** mDHFR (unfused DHFR) expressed in *E. coli* was extracted by sonication in 0.05M phosphate / 0.1M KCl / 2mM DTT, pH 8. The enzyme that did not bind to the Ni<sup>2+</sup>-NTA column was purified on a Methotrexate-Sepharose affinity column following the described procedure<sup>15</sup>. The enzyme was >95% pure as estimated by SDS-Page analysis and its specific activity was 10.3 U/mg.

**Analytical methods.** The purification of the fusion proteins was followed by sodiumdodecylsulfate-polyacrylamide (12%) gel electrophoresis according to the method of Lämmli<sup>16</sup>. Protein content was determined by the method of Lowry<sup>17</sup> using bovine serum albumin (BSA) as a standard. DHFR activity was measured as described by Buccanary et al.<sup>18</sup> The reduction of dihydrofolate

TABLE 1 Purification of DHFR-(His)<sub>2</sub>.

Sample	Volume (ml)	Protein (mg)	Activity (U)	Recovery (%)	Sp. Act. (U/mg)
Crude extract	189	3024	7306	100	2.4
Column flow-through	850	2258	704	9	0.3
Eluted DHFR-(His) <sub>2</sub>	270	714	6439	88	9.0
DHFR*					9.8
mDHFR**					10.3

30 g *E. coli* cells, recovered from 10 l fermentation broth were extracted by sonification in 180 ml 0.05 M sodium phosphate buffer, pH 8, containing 0.1 M potassium chloride, 0.1% Tween-20 and 10 µM PMSF. This crude extract was clarified by centrifugation and purified on the Ni<sup>2+</sup>-NTA column (Fig. 2).

\*DHFR prepared by carboxypeptidase A cleavage of DHFR-(His)<sub>2</sub>.

\*\*mDHFR (unfused DHFR) purified on a Methotrexate-Sepharose affinity column (see Experimental protocol).

TABLE 2 Affinity of poly-histidine fusion proteins for the Ni<sup>2+</sup>-NTA adsorbent in 6M GuHCl and 0.05M phosphate buffer.

Fusion protein	Phosphate		GuHCl	
	Retained (%)	Eluted (%)	Retained (%)	Eluted (%)
(His) <sub>2</sub> -DHFR	30	10	<5	—
(His) <sub>3</sub> -DHFR	90	75	<10	—
(His) <sub>4</sub> -DHFR	>90	30	10	10
(His) <sub>5</sub> -DHFR	>90	20	50	50
(His) <sub>6</sub> -DHFR	>90	10	>90	90
DHFR-(His) <sub>2</sub>	>90	90	<5	—
DHFR-(His) <sub>3</sub>	>90	80	<10	—
DHFR-(His) <sub>4</sub>	>90	50	10	10
DHFR-(His) <sub>5</sub>	>90	40	50	50
DHFR-(His) <sub>6</sub>	>90	30	>90	90
mDHFR*	<5	—	—	—

The proteins were extracted and chromatographed as exemplified for DHFR-(His)<sub>2</sub> and (His)<sub>6</sub>-DHFR. In GuHCl the products were eluted with a step gradient at pH 5, whereas in the phosphate system the products were eluted between pH 5.5 and 6.5 with a linear pH gradient from 8 to 5. The amount of extracted poly-histidine fusion protein was set = 100%. Protein was estimated on coomassie blue stained SDS Page. Crude extract prepared with 2 g of biomass was loaded onto a 15 ml column.

\*See Experimental Protocol

with NADPH was followed in 50 mM imidazole-HCl, pH 7 at 25°C. One unit converts 1  $\mu$ mole of dihydrofolate to tetrahydrofolate per minute. For amino acid analysis the instrumentation consisted of a Varian 5500 HPLC equipped with an *o*-phthalaldehyde post column derivatization device and a fluorescence detector. Amino-terminal amino acid sequence was determined with an Applied Biosystem 470 A Protein sequencer.

#### Acknowledgement

We wish to thank B. Auf der Maur, S. Kappey, V. Loechleiter, H. Taatjes, P. Jakob, G. Rummel and A. Schacher for excellent technical assistance. We are further grateful to R. Then for enzyme activity measurements, U. R  thlisberger for protein sequencing, M. Lanzer and H. Bujard for providing the promoter element and M.-L. Collino for typing the manuscript.

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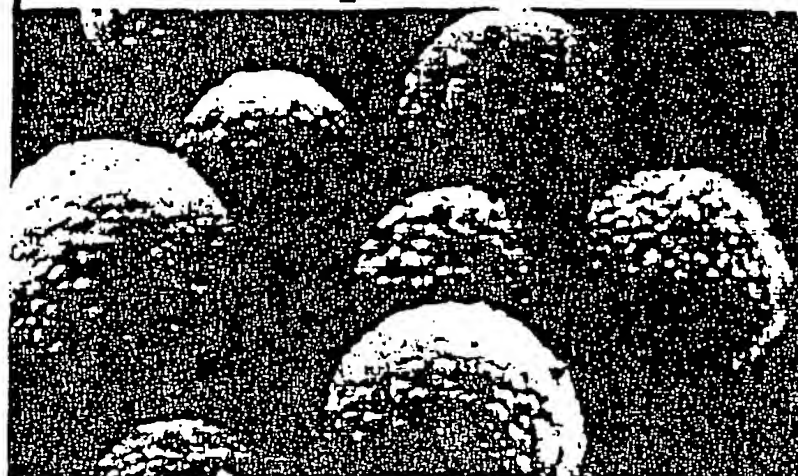
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#### Note added in proof

After our paper had been submitted, a similar fusion concept for the purification of proteins was published (Smith, M. C., Furman, T. C., Ingolia, T. D., Pidgeon, C. 1988. Chelating peptide-immobilized metal ion affinity chromatography. *J. Biol. Chem.* 263:7211-7215). For the purification of a hybrid protein comprising a His-Trp chelating peptide fused to proinsulin, immobilized iminodiacetic acid charged with Ni(II) ions (Ni<sup>2+</sup>-IDA adsorbent) was used. The IDA adsorbent is a tridentate chelate former, whereas the NTA adsorbent is a quadridentate chelate former that binds the metal ions more tightly, and has a high selectivity for adjacent histidine residues even in the presence of 6M GuHCl.

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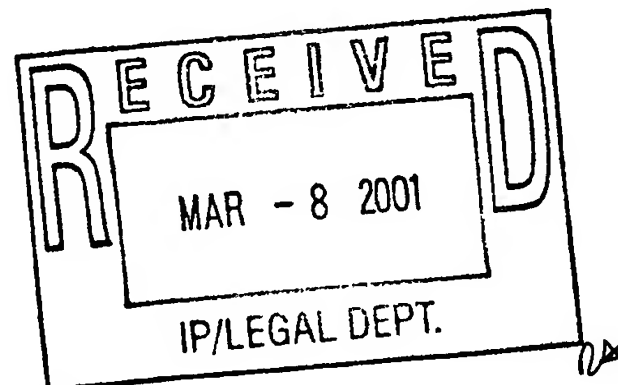
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## Notice of References Cited

Application No.  
09/351,414

Applicant(s)

Sheppard et al.

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